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COMPARISON BETWEEN OPTICAL COHERENCE TOMOGRAPHY TECHNIQUE AND MECHANICAL COMPRESSION ASSAY TO EVALUATE IONIZING RADIATION EFFECTS IN FROZEN AND LYOPHILIZED BONE TISSUE

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ABSTRACT

Currently tissue banks have utilized ionizing radiation to sterilize bone tissues to be used as allograft. This method is advantageous when compared with other techniques, because the tissue is sterilized in its final packaging avoiding later contaminations, another advantage is due to the fact occur only a minimal increase in temperature, in addition to provide a Sterility Assurance Level (SAL) of 10^{-6}, as recommended by national and international standards. However, there are several studies investigating the modifications that this method of sterilization may cause to the bone matrix, for example, alterations in the resistance to compression force. The compressive mechanical tests are highly used to evaluate the decrease in the mechanical strength; however it is a destructive assay. In this study, we used Optical Coherence Tomography to evaluate these possible changes. This technique is advantageous, for do not destroy the sample and enable the performing of other assays with the same sample. In literature, it is possible to find several studies about mechanical changes occasioned by destructive tests. Therefore, this study aims to compare the results of both techniques. It was selected four donors to obtain eight samples of fibula, through a partnership with the Tissue Bank (Instituto de Traumatologia do Hospital das Clínicas da Universidade de São Paulo). From each donor were separated twelve samples for preservation by freezing and twelve samples for preservation by lyophilization. The samples were analyzed by Optical Coherence Tomography (OCT) after irradiation at different doses (15, 25 and 50 kGy), in addition to non-irradiated control. After the samples were analyzed by Optical Coherence Tomography the same were subjected to mechanical testing. The data were analyzed by software developed by Dr. Anderson Zanardi de Freitas to calculate the total attenuation coefficient of photons. Nevertheless, only the preservation method may induce to alterations in the bone matrix, like with the lyophilized samples, in which a decrease of the attenuttion coefficient and mechanical resistance was verified, when compared to frozen samples. The lyophilized samples, probably, are the most sensitive to the effects of ionizing radiation, since the 25 kGy dose caused a significant reduction of the total optical attenuation coefficient: also, with this preservation method of preservation, the 50 kGy dose caused a decrease in the compressive stress.

1. INTRODUCTION

Tissues such as bone, cartilage, tendon, skin and amnion have been distributed by tissue banks worldwide for patients needing tissue transplantation. These institutions are responsible for donor selection, processing and distribution of tissues that will be used as allografts in reconstructive surgeries (1,2). In particular, bone is specially used to repair bone defects caused by illness or injury.

However, to avoid disease transmission from donor to recipients, tissues should be sterilized. ISO-11137:2006 (3) safety standard recommends a Sterility Assurance Level (defines the probability of a viable microorganism being present on an individual product unit after sterilization) of 10^{-6}, what implies that, in a sterilized tissue, only one live microorganism can be found in one million microorganisms. To achieve this goal, all processing steps follow good-practices standards, since donor selection to tissue storage. Tissue processing should be carried out aseptically and possible contamination should be reduced.

Ionizing radiation has been used as final sterilization because tissues can be sterilized in plastic bags developed, specifically, for tissue banks. Furthermore, this technique does not increase temperature significantly or leave toxic residues in the tissue (2).

Despite ionizing radiation advantages, there are some controversies regarding the dose that should be used to sterilize tissues, especially, bones. The main concern of tissue banks, surgeons and researchers is to know whether sterilization by ionizing radiation, as well as preservation methods, promote changes in tissues that could decrease their properties. For bones, it is particularly important to know how much the changes promoted by ionizing radiation sterilization affect the bone matrix, once these modifications may lead to a decrease in the mechanical properties, reducing, consequently, the allograft efficacy. (4, 5, 6).

In the "Radiation Sterilization of Tissue Allografts Practice Code: Requirements for Validation and Routine Control", produced by the International Atomic Energy Agency (IAEA), the dose recommended to sterilize tissues is 25 kGy. On other hand, several studies reported that doses greater than 20 kGy may cause bone matrix damages, reducing the mechanical properties (7). When ISO 11137 was revised in 2006, the minimal dose recommended for the sterilization of tissues was reduced to 15 kGy, considering an initial bio-burden equal to zero. In the literature, a trend to minimize the dose, using doses between 15 and 20 kGy, may be found (8).

The integrity of bone allografts does not depend only on mineral and collagen portions. It also depends on the interaction between these components in the bone matrix. Several studies showed that ionizing radiation can affect collagen structure (9).

Some non-destructive techniques can be used to evaluate possible changes in bone tissue. Optical Coherence Tomography (OCT) is a relative new technique that provides high-resolution images without prior preparation of the samples. This technique relies upon the properties of the backscattered photons from an infrared laser emission centered on the sample (10). In this work, OCT images associated with gold standards methods, such as mechanical tests, were used to verify if preservation and sterilization by ionizing radiation, in different doses, promote significant changes in tissues structures.

2. MATERIAL AND METHODS

2.1 Approval by the Ethics Committee

This project was submitted to the Committee of Ethics in Research, of the Faculdade de Saúde Pública da Universidade de São Paulo, and approved under the protocol n ° 2311 of January 31, 2012.

2.2 Samples collection

Eight fibulae were obtained from four human donors, through a partnership with Banco de Tecidos Musculo-esqueléticos (Bank of Muscle-Skeletal Tissues) of Instituto de Traumatologia e Ortopedia do Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo.

The age of donors ranged between 32 and 53 years old (mean 47). All donations by the Banco de Tecidos are in accordance with the determinations of ANVISA, described in a Board Resolution - RDC 220 of December 27, 2006, item 23.2 and the regulation of the National Transplant System (SNT), n ° 2600 of October 21, 2009, section 9, art. 156.

2.3 Processing of the samples

Samples were processed in three stages, named: mechanical processing, cuts and chemical processing. The mechanical processing consists of removal (with a scalpel) of adjacent tissues, such as blood remains, periosteum, subcutaneous tissue, muscle, fascia and fibrous tissue. After the mechanical processing, the fibulae epiphyses were removed from the shafts and the diaphises were transversely cut, in a parallel course to the longitudinal axis, by means of an electric hand saw, yielding 1 cm high rings.

After this procedure, the chemical processing was performed, when the bones were immersed in emulsifying solutions based on hydrogen peroxide and alcoholic solutions, under ultrasonic agitation. After these procedures, the samples were placed in sterile plastic bags containing saline solution and stirred manually, for approximately two minutes; 48 samples were obtained for freezing and 48 for lyophilization.

2.4 Samples Identification

The samples were identified according to the donor, type of preservation and the radiation dose received, as 0 (control), 15, 25 and 50 kGy.

2.5 Preservation

2.5.1 Lyophilization

The lyophilization, of the 48 processed samples, was carried out in an automated system composed of a lyophilization chamber (Labconco, Model 79480) and a condensation chamber (Labconco, Model 77530). The frozen samples, at -80 ° C, were placed within the lyophilization chamber at a temperature of -40 ° C.

Primary lyophilization was performed at a vacuum pressure of 8 x 10-3 mBar and a temperature of 30 ° C, for six hours. Subsequently, a secondary lyophilization was done at a temperature of 5 ° C and vacuum of 7 x 10-3 mBar. The limit established for residual moisture after lyophilization was up to 6%, measured at a moisture analyzer (Ohaus, Model MB45). For the experiments, the samples were rehydrated in saline for 10 minutes.

2.5.2 Freeze

After processing, 48 samples were frozen and stored in a -70° C freezer; before carrying out the tests, these samples were thawed at room temperature for 60 minutes.

2.6 Samples irradiation

The process of samples irradiation was carried out at the Centro de Tecnologia das Radiações (CTR) do Instituto de Pesquisas Energéticas e Nucleares (IPEN-CNEN/SP). The frozen and lyophilized samples were irradiated with gamma ray, by ^{60}Co sources in the compact Multipurpose Irradiator and received doses of 15 kGy, 25 kGy and 50 kGy, besides the non-irradiated control. For dose control, polymeric Red Perspex dosimeters (5-50 kGy) were used.

To prevent the variation in temperature, the samples frozen at -70 ° C were irradiated on dry ice and the lyophilized samples were irradiated in the presence of cooling elements. For each dose, twelve frozen samples and twelve lyophilized samples were irradiated, besides twelve

samples for each non- irradiated control, totaling 96 samples.

2.7 Optical Coherence Tomography

The OCT system uses a superluminescent LED (SLD) as optical source, with a spectral width of 100 ± 5 nm and centered at 930 nm, with a power of 2 mW on the sample. The images with lateral resolution of 6.0 microns and 4.1 microns longitudinal were generated by the displacement of the point of incidence on the sample by mirrors attached to a galvanometer. The light was focused on the sample with a lens of 5 cm focal distance. Data were acquired and stored in a microcomputer for later processing (OCP930SR, Thorlabs Inc. NJ, USA). Five images were obtained from each of the variables, for the subsequent calculation of the total optical attenuation coefficient.

2.7.1 Calculation of Total Optical Attenuation Coefficient

The coefficient calculation was performed by a dedicated computer program, developed by Dr. Anderson Zanardi de Freitas, in the platform LabView 8.0, National Instruments.
As it can be seen in the figure 1, the program allows a region of interest (ROI) to be selected, as observed at the top of the image, through the white and red lines. The ROI was, previously, determined and standardized for all samples, between 1500 and 4500 microns. After selecting the region of interest and placed the index of refraction of the bone tissue ($\eta = 1.56$), the program, automatically, calculates the optical attenuation coefficient.

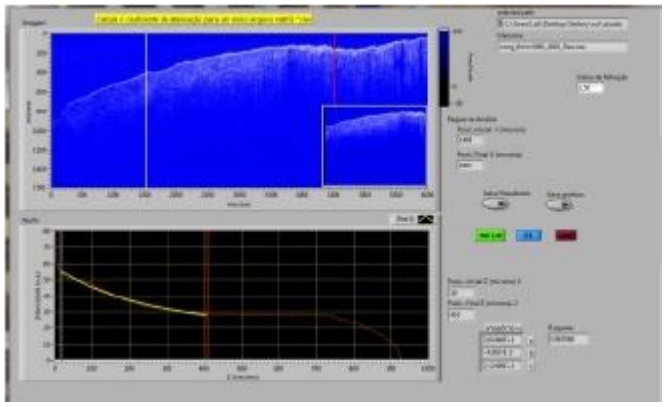

FIGURE 1 - Interface of the program used to calculate the total optical attenuation coefficient.

2.8 Compression Mechanical Assay

The compression tests were performed in an Instron Universal Testing Machine Model 5567, from Centro de Tecnologia das Radiações do Instituto de Pesquisas Energéticas e Nucleares (IPEN - CNEN / SP). This machine consists of an arrangement of two crossbars (one fixed and one mobile), a load cell, a direction finder and accessories to fix test bodies. The machine was connected to a computer and the assays were analyzed by Bluehil software.
The compression mechanical tests were performed at room temperature, using a load module of 10 kN. The ring-shaped samples, approximately 1 cm high, were pressed until there was a rupture in the material. Prior to testing, the samples were measured with a caliper, the samples had irregular shapes, however, three measurements were carried out in different regions of the sample and the average was calculated to obtain the measure of the internal and external diameters.

2.9 Statistical analysis

The results were compared, statistically, by ANOVA-Tukey method and those which presented statistical difference ($p < 0.05$) were marked on the graphs with the symbol (*).

3. RESULTS AND DISCUSSION

Solely by the OCT image, it is not possible to identify the structural modifications, once the generated images are constructed using values of the retro-scattering signal of tissue photons: from this signal, it is possible to calculate the total optical attenuation coefficient and, thus, to achieve a quantitative comparison among the different groups.

In 2012, Martinho Junior (11), used the total optical attenuation coefficient to evaluate the effects of preservation and radio-sterilization in human cartilage and verified that this greatness may be used for the prognosis of the tissue mechanical quality, since this measure is directly proportional to the rupture tension of the tissue.

In the histograms presented in figure 2, the results of the total optical attenuation coefficients mean of the bone tissue, preserved by different methods (freeze and lyophilizing) and iradiated in different doses (15, 25 e 50 kGy), can be observed.

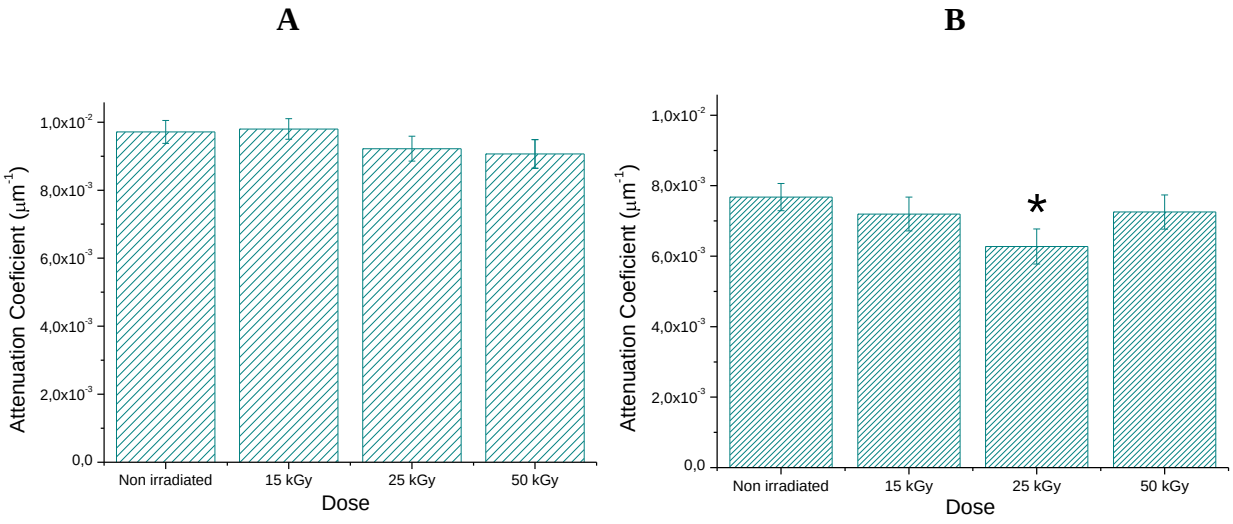

Figure 2**: A)** Graphs of the optical attenuation coefficients of the frozen, non irradiated samples and those irradiated with doses of 15, 25, and 50 kGy. **B)** Graphs of the optical attenuation coefficients of lyophilized, non irradiated samples and those irradiated with doses of 15, 25 and 50 kGy. (*) indicates the difference, statistically, significant related to the control.

With data obtained by the OCT technique on the bone tissue (FIGURA 2), it is observed a decrease of the total optical attenuation coefficient of the non irradiated control of the lyophilized samples, in relation to the non irradiated control of the frozen samples, revealing that the preservation method may induce to more harmful effects on the bone matrix than the irradiation itself.

This datum reinforces the study carried out by Komender, 1976, demonstrating that bones preserved by lyophilizing diminish by 35% the resistance to flexion, when compared to fresh bones (neither preserved nor irradiated).

The lyophilized samples, irradiated with a 25kGy dose, presented a difference statistically significant of the total optical attenuation coefficient, when compared to the non irradiated and lyophilized control.

According to Cara, 2012 (12), who used the OCT technique to detect the grades of dental enamel demineralization, during the development of cavity simulated lesions, the total optical attenuation coefficient of photons increase when they create empty spaces in the dental enamel structure, after the demineralization process, increasing the number of interfaces and, consequently, expanding the light spread.

The diminution of the total optical attenuation coefficient of photons on the bone tissue may be explained by a diminution of the spaces in the bone matrix, causing a decrease of interfaces with a consequent decrease of light scattering.

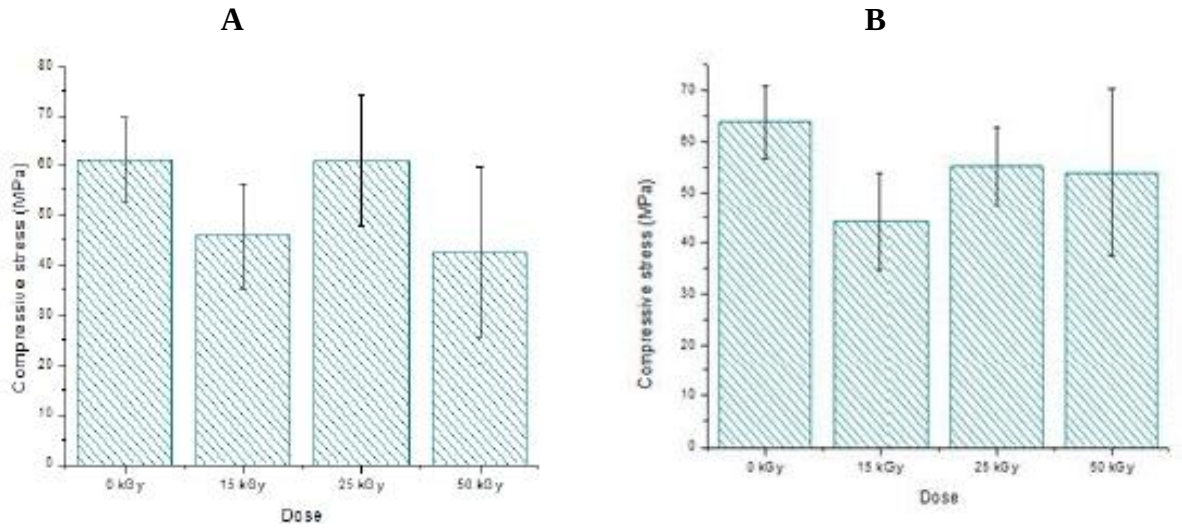

FIGURE 3 – **A)** Graph of the compressive stress mean, at the moment the sample breaks (MPa), frozen non irradiated and irradiated with doses of 15, 25 e 50 kGy. **B)** Graph of the compressive stress mean, at the moment the sample breaks (MPa), lyophilized non irradiated and irradiated with doses of 15, 25 e 50 kGy.

In figure 3 graph, it is observed that statistically significant differences did no occur, between the control frozen samples compared to the irradiated samples, with different doses. The same fact was observed with the lyophilized samples.

Nevertheless, it is observed that, despite it is not a significant difference, in both ways of preservation there was a greater reduction of the resistance to compression in the 15 kGy-irradiated samples and with the lyophilized samples with a 50 kGy dose.

In the compression test, a slight tendency to diminish the compression resistance was observed in the control lyophilized samples, since the mean of the compressive stress was 63.74 MPa in the control frozen samples, while in the lyophilized samples the mean of the compressive stress was 61.102 MPa: these data strengthen data found by the OCT.

Frozen samples, irradiated with a dose of 15Gy showed resistance to compression of 44.30 MPa and lyophilized samples, resistance of 45.82 MPa, with values relatively near in both preservation method. However, a reduction to compression resistance of 31% occurred with samples frozen and irradiated with 15 kGy, when compared to the respective control; as to lyophilized samples, the reduction was of 25%, compared to the respective control.

In 2005, Dziedzic-Goclawska et al. (9) demonstrated that, when dry by direct effect of radiation, the rupture of the collagen polipeptidic chain occurs with priority; when humid, by water radiolysis (direct effect), the intra and intermolecular cross of collagen chains may occur. Probably, with the dose of 15 kGy, it occurred, predominantly, the rupture of parts from some collagen chains, with the consequent decrease to compression resistance, since these samples had not been irradiated at room temperature (humid state).

With doses of 25 kGy, a decrease of 13% was observed in relation to the control frozen samples, while the lyophilized samples showed a decrease of 0.4%, compared to their control.

For the samples irradiated with a dose of 50 kGy, for both preservation method it was clear a decrease in the compression resistance, as the frozen samples (53.90 MPa) presented a fall of 15%, in relation to control, while the lyophilized samples (42.49 MPa) presented a fall of 30%, in relation to their control.

Probably, it may have occurred a constriction of the collagen propeller triple structure, what caused smaller spaces, that is, the light scattering diminished, leading to a reduction of the total optical attenuation coefficient. Nevertheless, this constriction of the collagen propeller triple structure causes a greater resistance of the bone tissue, when submitted to a compression mechanical assay.

From the results obtained in the present work, it may be concluded that the OCT techniques and the compression mechanical test were sensitive to detect alterations caused in the bone matrix, after preservation and radio-sterilization. According to the findings obtained, in both preservation method , the dose of 15 kGy was the one with a consequent diminution of the mechanical resistance.

Nevertheless, only the of preservation method may induce to alterations in the bone matrix, like with the lyophilized samples, in which a decrease of the attenuation coefficient and mechanical resistance was verified, when compared to frozen samples.

The lyophilized samples, probably, are the most sensitive to the effects of ionizing radiation, since the 25 kGy dose caused a significant reduction of the total optical attenuation coefficient: also, with this preservation method, the 50 kGy dose caused a decrease in the compressive stress.

4. CONCLUSION

From the results obtained in the present work, it may be concluded that the OCT techniques and the compression mechanical test were sensitive to detect alterations caused in the bone matrix, after preservation and radio-sterilization. According to the findings obtained, in both preservation method, the dose of 15 kGy was the one with a consequent diminution of the mechanical resistance.

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